

# Defective thermoregulation, impaired lipid metabolism, but preserved adrenergic induction of gene expression in brown fat of mice lacking C/EBP $\beta$

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C/EBP $\beta$  (CCAAT/enhancer-binding protein  $\beta$ ) is a transcriptional regulator of the UCP1 (uncoupling protein-1) gene, the specific marker gene of brown adipocytes that is responsible for their thermogenic capacity. To investigate the role of C/EBP $\beta$  in brown fat, we studied the C/EBP $\beta$ -null mice. When placed in the cold, C/EBP $\beta$ <sup>-/-</sup> mice did not maintain body temperature. This cold-sensitive phenotype occurred, although UCP1 and PGC-1 $\alpha$  (peroxisome-proliferator-activated receptor  $\gamma$  co-activator-1 $\alpha$ ) gene expression was unaltered in brown fat of C/EBP $\beta$ <sup>-/-</sup> mice. The UCP1 gene promoter was repressed by the truncated inhibitory C/EBP $\beta$  isoform LIP (liver-enriched transcriptional inhibitory protein, the truncated inhibitory C/EBP $\beta$  isoform). Since C/EBP $\beta$ -null mice lack both C/EBP $\beta$  isoforms, active LAP (liver-enriched transcriptional activatory protein, the active C/EBP $\beta$  isoform) and LIP, the absence of LIP may have a stronger effect than the absence of LAP upon UCP1 gene expression. Gene expression for UCP2 and UCP3 was not impaired in all tissues analysed. In primary brown adipocytes from C/EBP $\beta$ <sup>-/-</sup> mice, in-

duction of gene expression by noradrenaline was preserved. In contrast, the expression of genes related to lipid storage was impaired, as was the amount of triacylglycerol mobilized after acute cold exposure in brown fat from C/EBP $\beta$ <sup>-/-</sup> mice. LPL (lipoprotein lipase) activity was also impaired in brown fat, but not in other tissues of C/EBP $\beta$ <sup>-/-</sup> mice. LPL protein levels were also diminished, but this effect was independent of changes in LPL mRNA, suggesting that C/EBP $\beta$  is involved in the post-transcriptional regulation of LPL gene expression in brown fat. In summary, defective thermoregulation owing to the lack of C/EBP $\beta$  is associated with the reduced capacity to supply fatty acids as fuels to sustain brown fat thermogenesis.

**Key words:** brown fat, CCAAT/enhancer-binding protein  $\beta$  (C/EBP $\beta$ ), lipid metabolism, lipoprotein lipase, peroxisome-proliferator-activated receptor  $\gamma$  co-activator 1 $\alpha$  (PGC-1 $\alpha$ ), uncoupling protein (UCP).

## INTRODUCTION

C/EBPs (CCAAT/enhancer-binding proteins) encompass a family of transcription factors with structural and functional homologies. They contain basic leucine zipper domains, and are able to homo- and hetero-dimerize and to recognize common DNA-binding elements [1]. Several members of the C/EBP family (e.g. C/EBP $\alpha$ , C/EBP $\beta$  and C/EBP $\delta$ ) have tissue-restricted expression patterns and have been involved in the regulation of metabolic homeostasis [2,3]. C/EBP $\beta$  is most abundantly expressed in liver, BAT (brown adipose tissue) and WAT (white adipose tissue), reproductive tract and mammary gland [4–6]. Two C/EBP $\beta$  isoforms are generated from a single mRNA by regulated alternative translation from in-frame AUG codons [7], the full-length active LAP (liver-enriched transcriptional activatory protein, the active C/EBP $\beta$  isoform) and the truncated protein LIP (liver-enriched transcriptional inhibitory protein, the truncated inhibitory C/EBP $\beta$  isoform), which lacks the transactivation domain and behaves as a dominant inhibitory protein [8].

Mice with a deletion in the gene for C/EBP $\beta$  (C/EBP $\beta$ <sup>-/-</sup>) are born at the expected Mendelian ratio [9,10], but a subset of homozygous mice die of severe hypoglycaemia shortly after birth [11] owing to defective hepatic mobilization of glycogen and gluconeogenesis [12]. The surviving adult mice lacking C/EBP $\beta$  have a compromised immune system and impaired bactericidal activity of macrophages, and females are sterile [9,10]. In addition, adult

C/EBP $\beta$ <sup>-/-</sup> mice show decreased gluconeogenesis in liver and lipolysis in WAT during fasting and diabetes [13,14], but increased insulin sensitivity in skeletal muscle [15].

BAT is a major site for non-shivering thermogenesis in mammals in response to both cold exposure and diet. Thus transgenic mice with genetically ablated BAT are cold-intolerant and are prone to develop obesity [16]. Most of the thermogenic capacity of BAT is due to the presence of an inner mitochondrial protein uniquely expressed in brown adipocytes, the UCP (uncoupling protein), now referred to as UCP1, since the discovery of the more widely expressed UCP2 and UCP3 (for review see [17]). UCP1 uncouples oxidative phosphorylation from the respiratory chain, causing energy dissipation as heat [18], and mice with a deletion in the gene for UCP1 are cold-intolerant [19]. BAT thermogenic activity is mainly regulated by the sympathetic nervous system innervating the tissue. Thus noradrenaline promotes UCP1 gene expression mainly through the cAMP-mediated activation of UCP1 gene transcription [20]. It also induces both lipolysis and LPL (lipoprotein lipase)-mediated uptake of circulating triacylglycerols, thus providing fatty acids, the major metabolic fuel, for thermogenesis in the tissue. Moreover, fatty acids are direct activators of the uncoupling activity of UCP1 in the mitochondria [18]. In 1994, C/EBP $\alpha$  and C/EBP $\beta$  were identified as the first transcription factors involved in the regulation of UCP1 gene transcription [21] and were proposed to play a major role in the development of BAT [22]. Furthermore, the expression of

Abbreviations used: BAT, brown adipose tissue; C/EBP, CCAAT/enhancer-binding protein; FAS, fatty acid synthase; LAP, liver-enriched transcriptional activatory protein; LIP, liver-enriched transcriptional inhibitory protein; LPL, lipoprotein lipase; PEPCCK, phosphoenolpyruvate carboxykinase; PGC-1 $\alpha$ , peroxisome-proliferator-activated receptor  $\gamma$  co-activator-1 $\alpha$ ; PPAR $\gamma$ , peroxisome-proliferator-activated receptor  $\gamma$ ; UCP, uncoupling protein; WAT, white adipose tissue.

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C/EBP $\beta$ , but not C/EBP $\alpha$ , is enhanced in BAT after cold exposure [22]. While a critical role for C/EBP $\alpha$  in brown adipocyte differentiation has been demonstrated in mice with targeted deletion of the C/EBP $\alpha$  gene [23], the physiological role of C/EBP $\beta$  in BAT remains to be fully determined. Newborn C/EBP $\beta$ -deficient mice showed lower lipid accumulation in BAT than wild-type animals [24]. The expression of UCP1 mRNA in BAT from C/EBP $\beta$ <sup>-/-</sup> late foetuses was described to be decreased, although the expression of other adipogenic marker genes was unaltered [24].

In the present study, we report that C/EBP $\beta$ <sup>-/-</sup> mice are cold-intolerant. Although loss of C/EBP $\beta$  was expected to reduce UCP1 gene expression, present results indicate that UCP1 mRNA is increased in BAT of cold-exposed C/EBP $\beta$ <sup>-/-</sup> mice and in primary brown adipocytes from C/EBP $\beta$ <sup>-/-</sup> mice. In contrast, the present results on defective lipid metabolism in C/EBP $\beta$ <sup>-/-</sup> BAT, mainly the diminished LPL activity, point to an impaired supply of fatty acids as the metabolic fuel for BAT thermogenesis.

## EXPERIMENTAL

### Experimental animals

The care and use of mice were in accordance with the European Community Council Directive 86/609/EEC and were approved by the Comité Ètic d'Experimentació Animal of the University of Barcelona. The generation of the C/EBP $\beta$ -null mice has been described previously [9]. For the present study, C/EBP $\beta$ <sup>-/-</sup> mice were maintained in the C57BL6 genetic background and they were genotyped by Southern blot [9]. Mice were housed in micro-isolator cages and kept in standard conditions of housing (12 h: 12 h light/dark cycles), feeding (B. K. Universal diet, Barcelona, Spain) and environment temperature (21 ± 1 °C), unless otherwise indicated. Mice were 11–12 weeks old at the time of their use. To determine the effects of cold exposure, mice were exposed to 4 °C for 4 h. This time was chosen in order to conserve a representative population of C/EBP $\beta$ -null mice, as some individuals became hypothermic after 4 h at 4 °C (see the Results section). Body temperature was first measured at room temperature (21 °C) with an electronic thermistor equipped with a rectal probe. Mice were then transferred to a cold room at 4 °C, where the body temperature was measured every 1 h until the rectal temperature dropped to 27 °C.

### Cell culture and transfection assays

Primary culture of differentiated brown adipocytes was performed as described previously [25]. Precursor cells were isolated from the interscapular, cervical and axillary depots of BAT from either wild-type or C/EBP $\beta$ <sup>-/-</sup> 21-day-old mice. Experiments were performed on day 9 of culture, when 90% of the cells were considered to be differentiated on the basis of lipid accumulation and brown adipocyte morphology. Noradrenaline (Sigma) was added at 0.5 µM for 4 h.

The HIB-1B brown adipocyte cell line was cultured and transfected as reported previously [26]. The plasmid (–4551)UCP1-CAT contains the region –4551 to +110 of the rat UCP1 gene driving the promoterless chloramphenicol acetyltransferase gene. The expression vectors containing the entire open-reading frame of murine C/EBP proteins pMSV-C/EBP $\alpha$ , pMSV-C/EBP $\beta$  and pMEX-C/EBP $\delta$  were a gift from Dr S. McKnight (Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX, U.S.A.) and Dr P. Johnson (Laboratory of Protein Dynamics and Signaling, NCI-Frederick, Frederick, MD, U.S.A.) respectively. Dr U. Schibler (Department of Molecular Biology and NCCR Frontiers in Genetics, Sciences III, University of Geneva, Geneva, Switzerland) kindly provided the expression

vectors pCMV-LAP and pCMV-LIP, specific for either LAP or LIP. Each transfection contained 0.5 µg of UCP1-CAT, 0.2 µg of either C/EBP expression vector and 0.1 µg of cytomegalovirus  $\beta$ -galactosidase as internal control for variation in transfection efficiency. Enzymatic activities were measured as described previously [26].

### Northern blot analysis

Total RNA was isolated and Northern blot analysis was performed as reported previously [23]. Blots were stripped and rehybridized sequentially as required in each case. Autoradiographs were quantified by densitometric analysis using the Phoretics 1D software (Phoretics International).

### Preparation of protein extracts and Western blot analysis

BAT was homogenized in cold buffer A (5 mM Tes, pH 7.2, and 0.5 mM PMSF) containing 0.25 M sucrose. The homogenates were centrifuged at 1500 g for 10 min at 4 °C, and the pellet of nuclei was resuspended in buffer A (nuclear extract). The supernatant was then centrifuged at 8500 g for 10 min at 4 °C, and the pellet of mitochondria was resuspended in buffer A (mitochondrial extract). The final supernatant was the cytosolic/membrane extract. Western blot analysis was performed as reported previously [23]. Immunological detection was performed using antibodies directed against C/EBP $\alpha$  (a gift from Dr S. L. McKnight), C/EBP $\beta$  and C/EBP $\delta$  (C-19 and C-22; Santa Cruz Biotechnology), UCP1 (a gift from Dr E. Rial, Centro de Investigaciones Biológicas-CSIC, Madrid, Spain),  $\beta$ -actin (clone AC-15; Sigma), GLUT4 (a gift from Dr A. Zorzano, Department of Biochemistry and Molecular Biology, University of Barcelona, Barcelona, Spain) and LPL (a gift from Dr M. Robert, Department of Biochemistry and Molecular Biology, University of Barcelona, Barcelona, Spain).

### Assessment of brown fat composition and enzyme activities

Protein concentration was determined by the micromethod of Bio-Rad using BSA as standard. Total lipid extraction was performed on BAT [27], and triacylglycerol content was measured in the lipid extract using the GPO-Trinder reagent kit from Sigma. Cytochrome oxidase activity was measured as described previously [28]. Protein content and cytochrome oxidase activity were determined in both tissue homogenates and mitochondrial preparations. The calculated recovery of cytochrome oxidase activity in each sample was used to determine the amount of mitochondrial protein in the tissue. LPL activity was measured in tissue extracts and plasma as described previously [29]. One unit of enzyme activity was defined as the amount of enzyme that releases 1 µmol of oleate/min at 25 °C.

### Measurement of plasma metabolites

Blood was taken from the tail vein, and plasma was separated and frozen. Plasma triacylglycerol levels were measured with the GPO-Trinder reagent kit. Plasma concentrations of glucose and L-lactate were determined enzymatically using specific reagent kits from Sigma. Plasma non-esterified fatty acids were measured using a colorimetric acyl-CoA synthase/acyl-CoA oxidase-based method (Wako Chemicals).

### Statistical analysis

Where appropriate, statistical analysis was performed by Student's *t* test, and significance is indicated in the text.

**Table 1** Body mass, and WAT and BAT mass in wild-type and C/EBP $\beta^{-/-}$  mice

Values are means  $\pm$  S.E.M. for 14–20 11–12-week-old mice per group. They were pair-matched for litter origin and sex. Statistical differences compared with wild-type mice values are indicated: \* $P < 0.05$ ; \*\* $P < 0.01$ .

	Wild-type	C/EBP $\beta^{-/-}$
Body mass (g)	24.9 $\pm$ 0.4	22.6 $\pm$ 0.5**
Epididymal WAT mass (mg)	322.4 $\pm$ 40.8	204.7 $\pm$ 19.7*
Epididymal WAT mass/body mass (mg/g)	12.3 $\pm$ 1.4	8.8 $\pm$ 0.8*
BAT mass (mg)		
Interscapular	65.1 $\pm$ 7.1	48.0 $\pm$ 3.6*
Cervical	9.5 $\pm$ 0.7	7.1 $\pm$ 0.3**
Axillary	46.3 $\pm$ 3.7	34.5 $\pm$ 1.7**
BAT mass/body mass (mg/g)		
Interscapular	2.4 $\pm$ 0.15	2.0 $\pm$ 0.12*
Cervical	0.36 $\pm$ 0.02	0.30 $\pm$ 0.01**
Axillary	1.8 $\pm$ 0.08	1.5 $\pm$ 0.06**

**Table 2** Triacylglycerol and protein content, and cytochrome oxidase activity in BAT from wild-type and C/EBP $\beta^{-/-}$  mice

Values are means  $\pm$  S.E.M. for six to eight male mice per group. Statistical differences compared with wild-type mice values are indicated: \* $P < 0.05$ ; \*\* $P < 0.01$ .

	Unit	Wild-type	C/EBP $\beta^{-/-}$
Triacylglycerol	mmol/g of interscapular BAT	0.41 $\pm$ 0.03	0.39 $\pm$ 0.02
	$\mu$ mol/total interscapular BAT	26.8 $\pm$ 2.1	18.5 $\pm$ 0.9**
Protein	mg/g of interscapular BAT	85.4 $\pm$ 13.9	128.9 $\pm$ 14.0*
	mg/total interscapular BAT	5.5 $\pm$ 0.9	6.0 $\pm$ 0.7
Mitochondrial protein	mg/mg of protein	0.59 $\pm$ 0.06	0.45 $\pm$ 0.02*
	mg/total interscapular BAT	0.75 $\pm$ 0.05	0.76 $\pm$ 0.05
Cytochrome oxidase activity	$\mu$ mol/min per mg of protein	2.3 $\pm$ 0.2	1.7 $\pm$ 0.1**
	$\mu$ mol/min per mg of mitochondrial protein	4.7 $\pm$ 0.3	3.9 $\pm$ 0.2*
	$\mu$ mol/min per total interscapular BAT	12.8 $\pm$ 1.1	10.5 $\pm$ 0.5*

## RESULTS

### Reduced BAT in C/EBP $\beta^{-/-}$ mice is associated with lower triacylglycerol content

To analyse how C/EBP $\beta$  deletion affected BAT, we compared the body mass and fat depots of 11–12-week-old C/EBP $\beta^{-/-}$  and sex-matched wild-type littermates (Table 1). The average body mass was significantly lower in C/EBP $\beta^{-/-}$  mice, as was the amount of epididymal WAT as reported previously [13], even when expressed relative to body mass. In heterozygous mice, these parameters, as well as further results analysed in the present study, were similar to the wild-type ones (results not shown). The amount of the three BAT depots examined was significantly lower in C/EBP $\beta^{-/-}$  mice. When expressed relative to body mass, their mass remained significantly lower.

The decreased mass of BAT in C/EBP $\beta^{-/-}$  mice was associated with a significantly reduced triacylglycerol content, whereas the protein content was not modified (Table 2). Mitochondrial protein content was unaltered, but cytochrome oxidase activity was lower in the BAT of C/EBP $\beta^{-/-}$  mice than in wild-type controls, when expressed as specific activity or as total tissue activity, indicating a moderate decrease in the respiratory capacity of C/EBP $\beta^{-/-}$  BAT mitochondria.

### Thermoregulation is defective in C/EBP $\beta^{-/-}$ mice

The effects of C/EBP $\beta$  deficiency on thermoregulation were assessed by exposing mice to cold (4°C). Sensitivity to cold was measured as the percentage of mice that entered hypothermia, i.e. reduced their rectal temperature by 10°C. Mice were removed from the cold environment after a 10°C fall in temperature, otherwise they died. After only 4 h of cold exposure, 20% of C/EBP $\beta^{-/-}$  mice were found to be cold-sensitive, whereas none of the wild-type littermates showed this alteration. Longer periods of exposure to cold resulted in a higher percentage of C/EBP $\beta^{-/-}$  mice unable to maintain body temperature: 42% (24 h), 49% (48 h), 67% (72 h) and 80% (96 h) of the C/EBP $\beta$ -deficient mice entered hypothermia when kept at 4°C for up to 4 days. Only 10% of wild-type littermates were cold-sensitive after the longest period of time studied. Heterozygous mice were cold-resistant, similarly to wild-type mice. Males and females did not show significant differences in cold sensitivity.

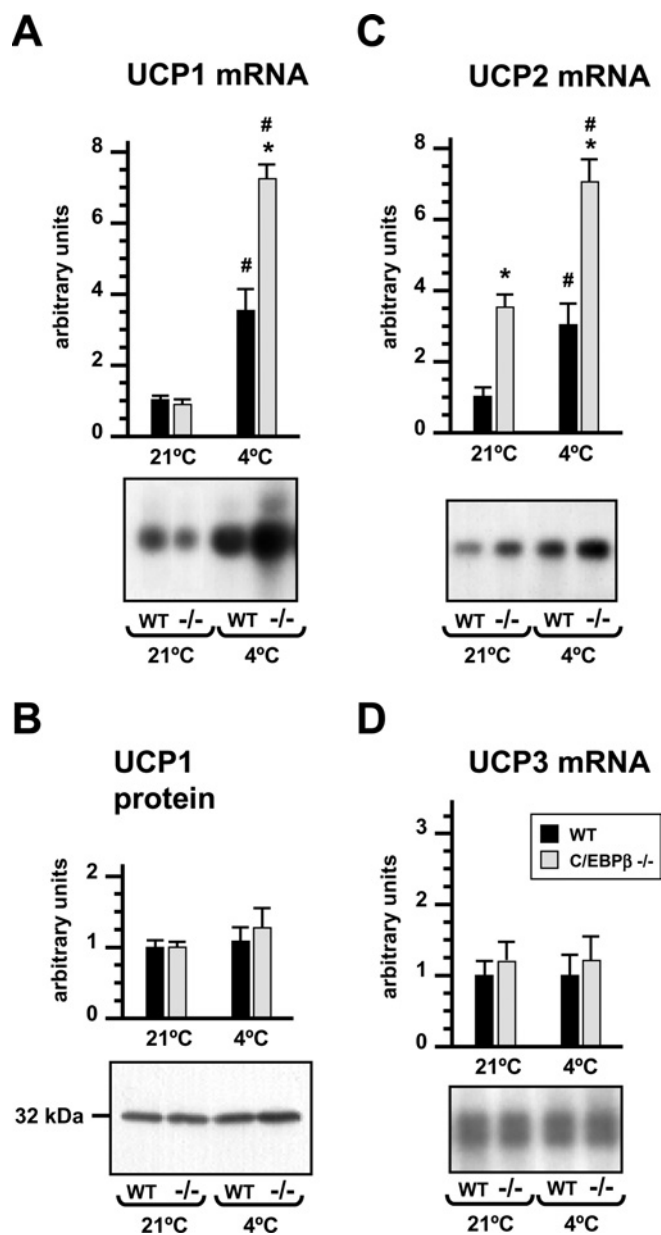
### The induction of UCP1 gene expression by cold exposure is not impaired in the brown fat of C/EBP $\beta^{-/-}$ mice

As shown in Figure 1(A), the mRNA levels for UCP1 were similar in BAT from wild-type and C/EBP $\beta^{-/-}$  control mice at 21°C. Acute cold exposure (4 h at 4°C) significantly increased UCP1 mRNA levels in wild-type mice, as reported previously [30]. UCP1 mRNA levels were significantly higher in cold-exposed C/EBP $\beta^{-/-}$  mice than in wild-type mice. The levels of UCP1 protein did not differ between wild-type and C/EBP $\beta^{-/-}$  mice (Figure 1B). Exposure to cold (4 h at 4°C) did not significantly increase the content of UCP1 in either type of mice, as expected for such a short-term exposure to cold owing to the known time delay between the increase in UCP1 mRNA levels and that of UCP1 protein in response to cold [31]. The subset of C/EBP $\beta^{-/-}$  mice which did not enter hypothermia after 4 days at 4°C was chosen to analyse the effects of longer exposure to cold. Under these conditions, wild-type mice showed increased UCP1 mRNA (4.2  $\pm$  0.4-fold) and UCP1 protein (2.4  $\pm$  0.3-fold) with respect to mice kept at 21°C. As a result of this longer exposure to cold, C/EBP $\beta^{-/-}$  mice showed a significantly higher induction of UCP1 mRNA (2.4  $\pm$  0.2-fold increase in C/EBP $\beta^{-/-}$  compared with wild-type, both at 4°C for 4 days;  $P < 0.05$ ) and of UCP1 protein levels (2.1  $\pm$  0.2-fold increase in C/EBP $\beta^{-/-}$  compared with wild-type, both at 4°C for 4 days;  $P < 0.05$ ).

### The expression of UCP2 and UCP3 is not impaired in mice lacking C/EBP $\beta$

UCP2 mRNA levels were significantly up-regulated in C/EBP $\beta^{-/-}$  compared with wild-type control mice at 21°C (Figure 1C). UCP2 mRNA levels in BAT were also increased by cold exposure as described in [32], but higher levels were reached in C/EBP $\beta^{-/-}$  mice. The lack of C/EBP $\beta$  did not modify the mRNA levels for UCP3 (Figure 1D). UCP3 mRNA expression was not sensitive to cold exposure in wild-type, in agreement with previous reports [32], or in C/EBP $\beta^{-/-}$  mice.

To test whether the up-regulation of UCP2 mRNA due to the lack of C/EBP $\beta$  was specific for BAT, UCP2 mRNA expression was determined in WAT, heart and spleen. All the tissues tested revealed overexpression of UCP2 mRNA in C/EBP $\beta^{-/-}$  compared with wild-type mice (results not shown), indicating that the up-regulation of UCP2 mRNA was not specific to BAT, but is an overall phenomenon that is likely to be caused by indirect mechanisms, as occurred in tissues characterized by either high (BAT, WAT and spleen) or low (heart) expression of C/EBP $\beta$ . This effect



**Figure 1** Effects of cold exposure on uncoupling protein gene expression in brown fat from wild-type and C/EBP $\beta^{-/-}$  mice

(A), (C) and (D) Northern blot analyses of total RNA extracted from the interscapular BAT depot of wild-type (WT) or homozygous C/EBP $\beta^{-/-}$  mice at room temperature (control; 21°C) or after cold exposure to 4°C for 4 h (4°C). Data are expressed as a percentage of the wild-type value, which was set to 1. Results are means  $\pm$  S.E.M. for three to four independent analyses, each performed by comparing littermates. Statistical significance of comparison between genotypes is shown as  $*P \leq 0.05$ , and between control and cold-exposed mice as  $\#P \leq 0.05$ . A representative Northern blot analysis is depicted beneath the histograms. (B) Immunoblot analysis of mitochondrial protein extracts isolated from interscapular BAT of control or cold-exposed wild-type (WT) or C/EBP $\beta^{-/-}$  mice as described above. Results are means  $\pm$  S.E.M. for three independent analyses, each performed by comparing littermates. A representative Western blot analysis is depicted, and the size of the signal obtained in kDa is shown to the left of the blot.

may be related to the described role of UCP2 in macrophage-mediated immunity [33], probably in compensation for the impaired capacity to activate macrophages in C/EBP $\beta^{-/-}$  mice [9,10]. However, when the levels of UCP2 protein were assessed using a highly specific antibody, no differences between genotypes were found in any tissue (F. Villarroya, B. Miroux

and D. Ricquier, unpublished work), which is consistent with the complex post-transcriptional mechanisms of UCP2 gene regulation [34]. UCP3 mRNA levels in skeletal muscle, the major site, together with BAT, of UCP3 gene expression, were unaltered in C/EBP $\beta^{-/-}$  mice (results not shown).

#### The expression of adipogenic marker genes is unaltered in BAT of C/EBP $\beta^{-/-}$ mice

The mRNA levels for PEPCK (phosphoenolpyruvate carboxy-kinase), aP2/aFABP (cytosolic fatty-acid-binding protein) and the glucose transporter GLUT4, marker genes of adipose differentiation whose gene promoters are targets of C/EBP transcription factors, were unaltered in BAT from C/EBP $\beta^{-/-}$  mice (Figure 2A). The abundance of GLUT4 protein was also unaltered in the BAT of C/EBP $\beta^{-/-}$  mice (results not shown). In contrast, the expression of the FAS (fatty acid synthase) gene was significantly decreased in the BAT of C/EBP $\beta^{-/-}$  mice. Cold exposure also down-regulated FAS mRNA expression, as was found in C/EBP $\beta^{-/-}$  mice.

The expression of the PPAR $\gamma$  (peroxisome-proliferator-activated receptor  $\gamma$ ), a master regulator of adipose differentiation, was not modified in the BAT of C/EBP $\beta^{-/-}$  mice (Figure 2B). Cold exposure tended to decrease PPAR $\gamma$  mRNA levels, although the difference was not significant. No differences were observed between genotypes.

We next analysed the expression of the PGC-1 $\alpha$  (PPAR $\gamma$  co-activator-1 $\alpha$ ) gene, as it plays a critical role in co-ordinating the BAT adaptive thermogenic response at the transcriptional level [35]. As shown in Figure 2(C), levels of PGC-1 $\alpha$  mRNA were dramatically increased upon cold exposure, as described previously [35]. The expression of PGC-1 $\alpha$  mRNA in the BAT of C/EBP $\beta^{-/-}$  mice was similar to that in wild-type littermates.

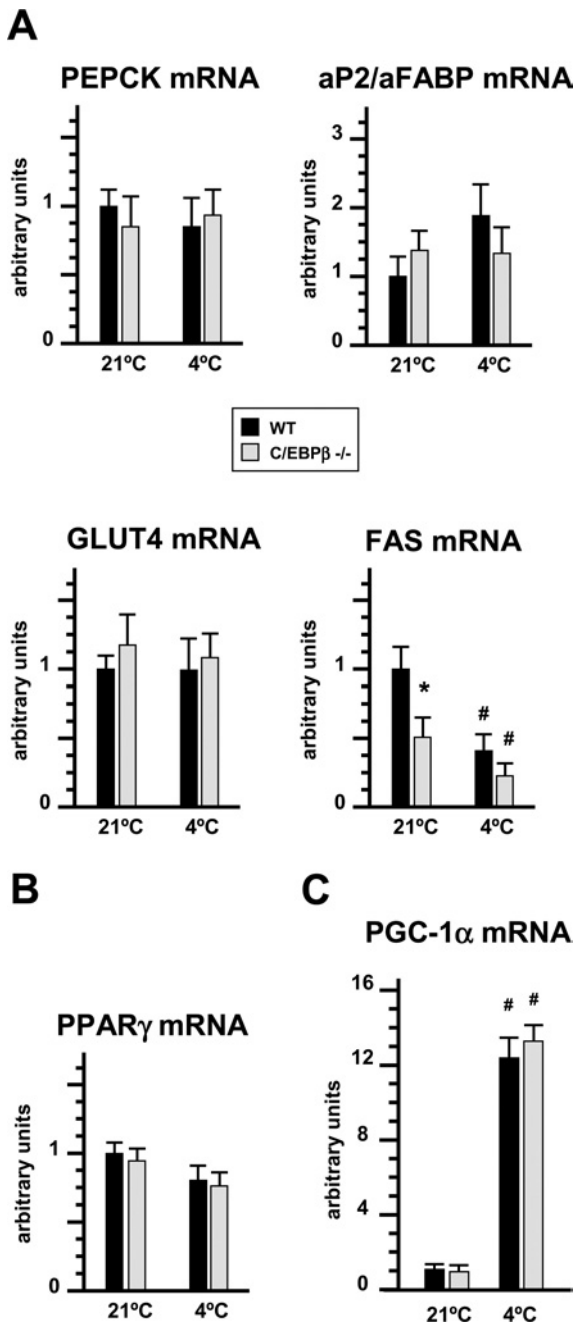
Thus the lack of C/EBP $\beta$  did not alter the expression of gene markers of adipose differentiation in BAT, including UCP1, the brown-fat-specific gene responsible for adaptive thermogenesis, and only the expression of FAS mRNA was impaired.

#### Expression of neither C/EBP $\alpha$ nor C/EBP $\delta$ increased in BAT of C/EBP $\beta^{-/-}$ mice

The expression of C/EBP $\alpha$  and C/EBP $\delta$  mRNA was not modified in C/EBP $\beta^{-/-}$  mice (Figures 3A and 3C). Furthermore, acute cold exposure significantly increased C/EBP $\delta$  mRNA in the BAT of mice from both genotypes, whereas C/EBP $\alpha$  mRNA levels tended to be decreased. Cold exposure significantly reduced the protein content of C/EBP $\alpha$  in both genotypes (Figure 3B). C/EBP $\delta$  amount did not differ between genotypes either (Figure 3D). Thus the lack of C/EBP $\beta$  was not compensated by increased expression of C/EBP $\alpha$  or C/EBP $\delta$  in BAT.

#### The induction of gene expression by noradrenaline is preserved in C/EBP $\beta^{-/-}$ primary brown adipocytes

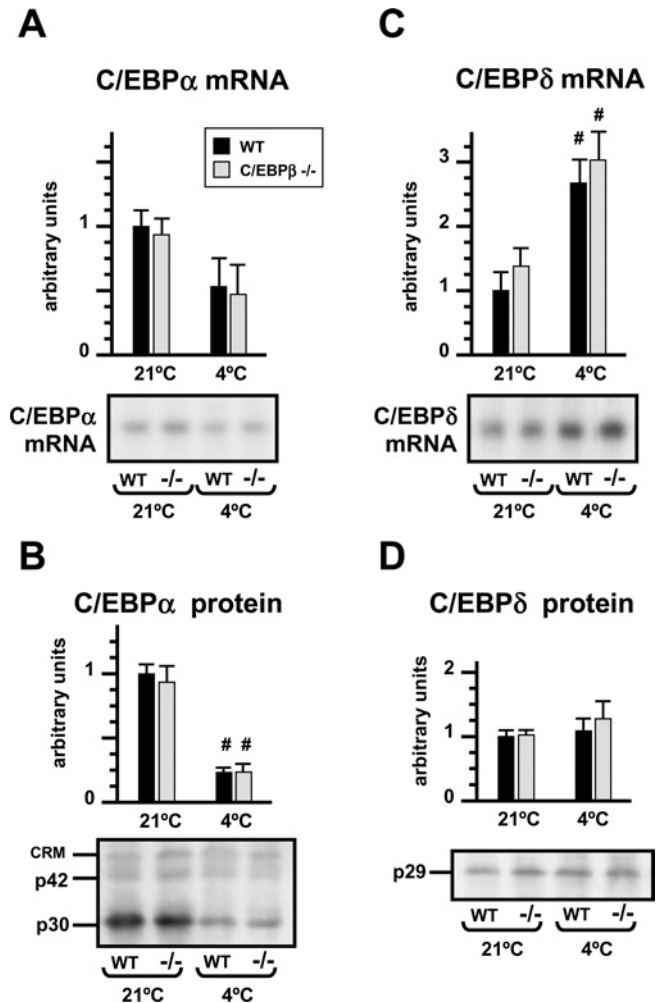
To determine whether gene expression in BAT from C/EBP $\beta^{-/-}$  mice was characteristic of differentiated brown adipocyte cells and not due to extra-tissue effects, we performed primary cultures of brown adipocytes. Cells differentiated in culture from precursor cells were obtained from either wild-type or C/EBP $\beta^{-/-}$  BAT. No major differences in the differentiation process were observed. When gene expression was analysed on day 9 of culture (fully differentiated brown adipocytes), C/EBP $\beta^{-/-}$  cells showed a slightly diminished lipid content in direct correlation with decreased mRNA expression of genes for lipid metabolism such as aP2/aFABP ( $0.45 \pm 0.12$  in comparison with the wild-type cell value which was set to 1;  $P < 0.05$ ), FAS ( $0.48 \pm 0.09$ ;  $P < 0.05$ )



**Figure 2** Effects of cold exposure on the expression of adipogenic marker genes in brown fat from wild-type and C/EBP $\beta$ <sup>-/-</sup> mice

(A) mRNA levels in brown fat of the indicated metabolic genes. (B) and (C) Expression of the mRNA for PPAR $\gamma$  (B) and for the thermogenic co-activator PGC-1 $\alpha$  (C) as evaluated by Northern blotting. For experimental and statistical details, see the legend to Figure 1.

and LPL ( $0.70 \pm 0.09$ ;  $P < 0.05$ ), whereas the expression of PEPCK and GLUT4 mRNAs was unaffected ( $0.94 \pm 0.11$  and  $1.06 \pm 0.08$  respectively). In contrast, UCP1 mRNA levels were significantly increased in C/EBP $\beta$ <sup>-/-</sup> brown adipocytes (Figure 4A). Treatment of cells with  $0.5 \mu\text{M}$  noradrenaline for 4 h significantly induced UCP1 and PGC-1 $\alpha$  gene expression, which was preserved in C/EBP $\beta$ <sup>-/-</sup> cells. Likewise, the decrease in PPAR $\gamma$  mRNA levels by noradrenaline, as reported recently [36], was also preserved in C/EBP $\beta$ <sup>-/-</sup> cells. The expression of

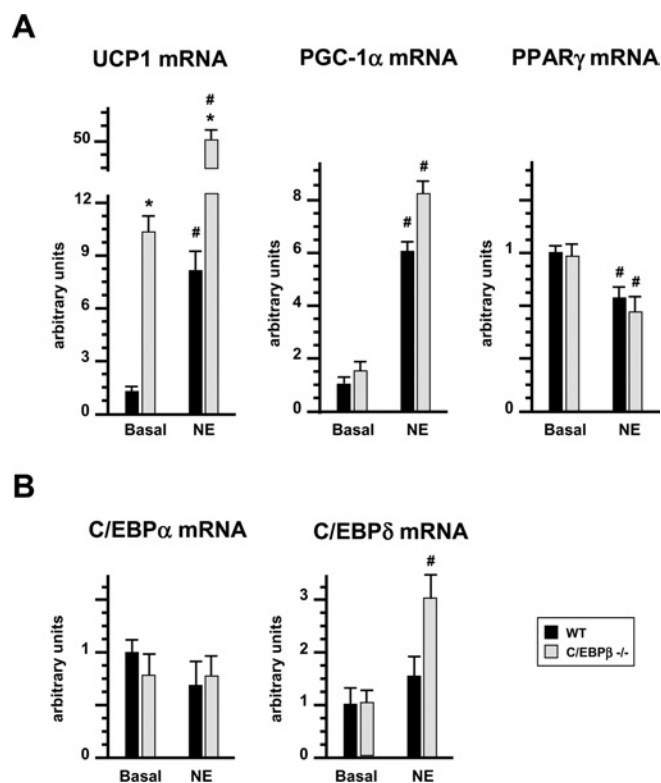


**Figure 3** Effects of cold exposure on the expression of genes for C/EBP $\alpha$  and C/EBP $\delta$  in brown fat from wild-type and C/EBP $\beta$ <sup>-/-</sup> mice

For experimental and statistical details, see the legend to Figure 1. (A) and (C) Northern blot analysis. (B) and (D) Immunoblot analysis of nuclear protein extracts isolated from interscapular BAT from wild-type (WT) or C/EBP $\beta$ <sup>-/-</sup> mice, exposed or not to 4°C for 4 h. The sizes of the signals obtained and the position of cross-reactive molecules (CRM) are shown to the left of the blots.

C/EBP $\alpha$  mRNA was unaltered, whereas C/EBP $\delta$  mRNA levels were increased by noradrenaline only in C/EBP $\beta$ <sup>-/-</sup> adipocytes (Figure 4B).

Since the deletion of the gene for C/EBP $\beta$  abolishes not only the expression of LAP, but also that of LIP, the increased expression of the C/EBP $\beta$ -target gene UCP1 may be due to the lack of negative LIP rather than of active LAP. The relative abundance of full-length C/EBP $\beta$  in liver and brown fat of wild-type mice was similar, but the amount of LIP was high in BAT and barely detectable in liver (Figure 5A), in agreement with previous reports in rat [22]. Transient transfection experiments were performed to analyse whether the UCP1 gene promoter is transactivated by C/EBP isoforms. As reported previously [21], both C/EBP $\alpha$  and C/EBP $\beta$  transactivated the UCP1 gene promoter (Figure 5B). C/EBP $\delta$  showed a similar capacity to induce UCP1 gene transcription. When expressed independently, LAP was 4-fold more active than C/EBP $\beta$ , whereas LIP blocked the C/EBP $\alpha$ -dependent transactivation of the UCP1 gene promoter. Thus a major role of LIP as a transcriptional inhibitor of the UCP1 gene is established.



**Figure 4** Expression of genes for UCP1, co-activator PGC-1 $\alpha$ , PPAR $\gamma$  and C/EBPs in primary brown adipocytes from wild-type and C/EBP $\beta$ <sup>-/-</sup> mice

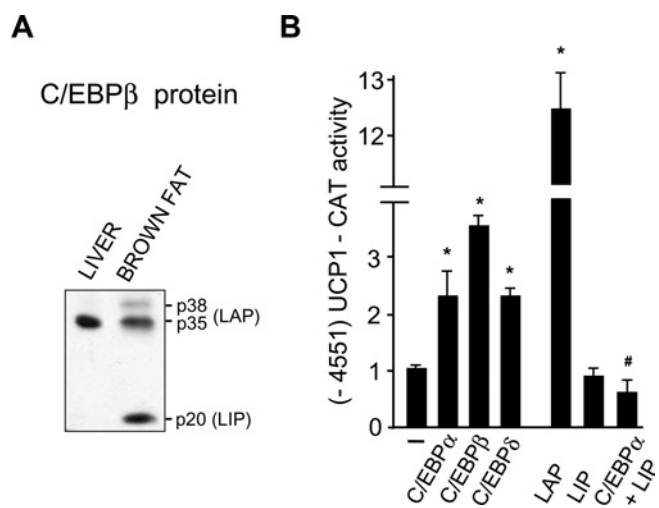
Brown adipocytes differentiated in culture from precursor cells isolated from BAT from wild-type (WT) or C/EBP $\beta$ <sup>-/-</sup> mice (day 9 of culture) were exposed (NE) or not (basal) to 0.5  $\mu$ M noradrenaline for 4 h. Northern blot analyses were performed with 10  $\mu$ g of total RNA extracted from three pooled plates. Results are means  $\pm$  S.E.M. for three independent experiments on various cultures and are expressed relative to the untreated wild-type cells, which were set to 1. Statistical significance of comparison between genotypes is shown as \* $P$   $\leq$  0.05, and between basal and noradrenaline-treated cells as # $P$   $\leq$  0.05.

#### Effects of cold exposure on plasma metabolites in C/EBP $\beta$ <sup>-/-</sup> mice

When kept at 21 °C, C/EBP $\beta$ <sup>-/-</sup> mice showed lower levels of plasma glucose and non-esterified fatty acids, increased lactate and similar triacylglycerol levels to those of wild-type littermates (Table 3), as described previously [12–14]. When wild-type mice were exposed to 4 °C for 4 h, plasma glucose and non-esterified fatty acid levels rose, whereas lactate and triacylglycerol concentrations were not modified. The rise in plasma glucose due to 4 h of cold was moderate in wild-type mice (approx. 20 %), but much stronger in C/EBP $\beta$ <sup>-/-</sup> mice (approx. 70 %). Non-esterified fatty acid levels were also increased as a consequence of cold exposure in C/EBP $\beta$ <sup>-/-</sup> mice, but to an extent similar to that found in wild-type mice. Lactate was not affected by cold in C/EBP $\beta$ <sup>-/-</sup> mice, and, in contrast with wild-type, C/EBP $\beta$ <sup>-/-</sup> mice showed a significant rise in plasma triacylglycerol levels when exposed to cold.

#### Reduced triacylglycerol store mobilization by cold exposure in brown fat of C/EBP $\beta$ <sup>-/-</sup> mice

To test whether the lack of C/EBP $\beta$ <sup>-/-</sup> affects the capacity of stored triacylglycerols to be mobilized in response to thermogenic requirements, the effects of cold on triacylglycerol content in BAT were determined. After 4 h of cold exposure, a similar reduction (approx. 60 %) in the BAT triacylglycerol content was found in both genotypes [wild-type, 27.9  $\pm$  2.3  $\mu$ mol/total interscapular



**Figure 5** Effects of C/EBP co-transfection on the expression of the UCP1 gene promoter

(A) Immunoblot analysis of nuclear protein extracts isolated from liver or interscapular BAT from wild-type mice. Results are representative of two separate experiments. The sizes of the signals obtained are shown to the right of the lanes. (B) HIB-1B cells were co-transfected with 0.5  $\mu$ g of (–4551)UCP1-CAT along with 0.2  $\mu$ g of expression vector for C/EBP $\alpha$ , C/EBP $\beta$  or C/EBP $\delta$ , or the expression vectors specific for either LAP (active full-length C/EBP $\beta$ ) or LIP (truncated inhibitory C/EBP $\beta$ ), as indicated. pCMV- $\beta$ galactosidase (0.1  $\mu$ g) was added to each transfection as an internal control for transfection efficiency. Results are expressed as fold change compared with those of control cells, and are the means for two to three independent experiments, each performed in duplicate. Statistical significance of comparison between basal and C/EBP co-transfected cells is shown as \* $P$   $\leq$  0.05, and between C/EBP $\alpha$  alone and C/EBP $\alpha$  plus LIP as # $P$   $\leq$  0.05.

**Table 3** Plasma glucose, lactate, non-esterified fatty acid and triacylglycerol levels in wild-type and C/EBP $\beta$ <sup>-/-</sup> mice

Plasma metabolite levels were measured in the fed state at room temperature (21 °C) or after 4 h at 4 °C. Results are means  $\pm$  S.E.M. of six to eight male mice per group. Statistical differences between wild-type and C/EBP $\beta$ -null mice at the same temperature are indicated: \* $P$  < 0.05; \*\* $P$  < 0.01. Significance between control and cold-exposed animals within the same genotype: # $P$  < 0.05; ## $P$  < 0.01.

	Wild-type		C/EBP $\beta$ <sup>-/-</sup>	
	21 °C	4 °C	21 °C	4 °C
Glucose (mM)	12.3 $\pm$ 0.5	14.6 $\pm$ 0.5##	9.7 $\pm$ 0.6**	16.5 $\pm$ 1.8##
Lactate (mM)	3.3 $\pm$ 0.2	2.9 $\pm$ 0.2	4.4 $\pm$ 0.4*	4.9 $\pm$ 0.6**
Non-esterified fatty acids (mM)	0.58 $\pm$ 0.03	0.87 $\pm$ 0.04##	0.46 $\pm$ 0.04*	0.78 $\pm$ 0.16#
Triacylglycerols (mM)	9.0 $\pm$ 0.7	8.2 $\pm$ 0.4	9.1 $\pm$ 0.7	11.2 $\pm$ 1.5*

BAT (21 °C) to 10.5  $\pm$  1.7  $\mu$ mol/total interscapular BAT (4 °C at 4 h); C/EBP $\beta$ <sup>-/-</sup>, 17.8  $\pm$  1.0  $\mu$ mol/total interscapular BAT (21 °C) to 6.2  $\pm$  1.2  $\mu$ mol/total interscapular BAT (4 °C at 4h)], indicating that the activation of lipolysis is unaffected in C/EBP $\beta$ <sup>-/-</sup> mice. In contrast, the absolute amount of triacylglycerols mobilized due to cold in C/EBP $\beta$ <sup>-/-</sup> mice (11.6  $\mu$ mol of triacylglycerol) was only two-thirds of the value in wild-type mice (17.4  $\mu$ mol of triacylglycerol), thus indicating a reduction of the overall endogenous fuel available for BAT thermogenesis in C/EBP $\beta$ <sup>-/-</sup> mice.

#### Reduced basal and cold-induced LPL activity in brown fat of C/EBP $\beta$ <sup>-/-</sup> mice

The activity of LPL, the major source of supply of circulating fatty acids to sustain fuel oxidation for thermogenesis in BAT,

**Table 4** Effect of cold exposure on LPL activity in BAT, WAT, skeletal muscle, heart and plasma from wild-type and C/EBP $\beta^{-/-}$  mice

The activity of LPL was determined in the interscapular BAT, epididymal WAT, tibialis anterior skeletal muscle, heart and plasma as outlined in the Experimental section. Values are means  $\pm$  S.E.M. of six to eight male mice per group. Statistical differences between wild-type and C/EBP $\beta$ -null mice at the same temperature are indicated: \* $P < 0.05$ ; \*\* $P < 0.01$ . Significance between control and cold-exposed animals within the same genotype: ## $P < 0.01$ .

LPL activity		Wild-type		C/EBP $\beta^{-/-}$	
		21°C	4°C	21°C	4°C
m-units/g of tissue	Interscapular BAT	355.5 $\pm$ 40.3	775.3 $\pm$ 29.4 <sup>##</sup>	288.2 $\pm$ 23.1	425.4 $\pm$ 31.4 <sup>***</sup>
	WAT	265.5 $\pm$ 35.4	149.2 $\pm$ 8.8 <sup>##</sup>	227.0 $\pm$ 37.2	163.0 $\pm$ 10.7
	Skeletal muscle	37.0 $\pm$ 7.3	32.0 $\pm$ 1.0	43.3 $\pm$ 2.9	55.3 $\pm$ 9.8
	Heart	272.6 $\pm$ 25.8	285.5 $\pm$ 35.5	263.7 $\pm$ 17.9	254.5 $\pm$ 22.5
m-units/mg of protein	Interscapular BAT	7.2 $\pm$ 0.8	16.5 $\pm$ 1.5 <sup>##</sup>	5.5 $\pm$ 0.4*	7.2 $\pm$ 1.1**
	WAT	11.0 $\pm$ 1.8	10.0 $\pm$ 1.5	8.0 $\pm$ 1.4	7.8 $\pm$ 0.9
	Skeletal muscle	1.0 $\pm$ 0.2	0.7 $\pm$ 0.1	0.8 $\pm$ 0.1	1.2 $\pm$ 0.3
	Heart	4.8 $\pm$ 0.4	4.2 $\pm$ 0.5	4.7 $\pm$ 0.3	5.0 $\pm$ 0.5
m-units/ml of plasma		0.55 $\pm$ 0.05	0.65 $\pm$ 0.11	0.44 $\pm$ 0.03	0.40 $\pm$ 0.06*

was determined in basal conditions and after cold exposure in BAT and other peripheral tissues from wild-type and C/EBP $\beta^{-/-}$  mice.

The lack of C/EBP $\beta$  did not significantly alter LPL activity in WAT, skeletal muscle or heart when mice were kept at 21°C, when enzyme activity was expressed per g of tissue or per mg of protein (Table 4). LPL activity in plasma was also unchanged. In contrast, it was significantly reduced when expressed per mg of protein in BAT from C/EBP $\beta^{-/-}$  compared with wild-type mice. Cold exposure of wild-type mice significantly enhanced LPL activity in BAT, but not in any other tissue, in agreement with previous reports [37]. The rise in LPL activity in BAT due to cold was impaired in C/EBP $\beta^{-/-}$  mice. Longer exposure to cold (15 h) still resulted in higher LPL specific activity in BAT of wild-type (15.4 m-units/mg of protein) compared with surviving C/EBP $\beta^{-/-}$  mice (6.5 m-units/mg of protein;  $P < 0.05$ ). LPL activity was also impaired in mice kept at 21°C and in those exposed to 4°C when expressed per whole interscapular BAT depot, as an index of the overall capacity of the tissue for fatty acid uptake (Figure 6C). However, when LPL RNA was determined, C/EBP $\beta^{-/-}$  and wild-type mice did not differ in basal or in cold-exposed conditions (Figure 6A), when LPL mRNA abundance was doubled in both genotypes. In contrast, LPL protein mass decreased paralleling changes in activity (Figure 6B), indicating that the lack of C/EBP $\beta$  affects LPL activity at a post-transcriptional level.

## DISCUSSION

Mice homozygous for a deletion of the gene for C/EBP $\beta$  have been reported to show impaired macrophage activity and altered glucose homeostasis [9–14]. In the present study, we establish that C/EBP $\beta^{-/-}$  mice are cold-intolerant. The production of heat by non-shivering thermogenesis in BAT is required to maintain body temperature in rodents. UCP1 plays a crucial role in this process, and mice with targeted inactivation of this gene are intolerant to cold [19]. Adrenergic regulation of BAT is also required, since mice deficient in noradrenaline by targeted deletion of the dopamine  $\beta$ -hydroxylase gene also have defective thermoregulation [38]. However, present results indicate that C/EBP $\beta^{-/-}$  mice are cold-intolerant, although neither UCP1 expression nor the adrenergic-mediated responses to cold are impaired in BAT.

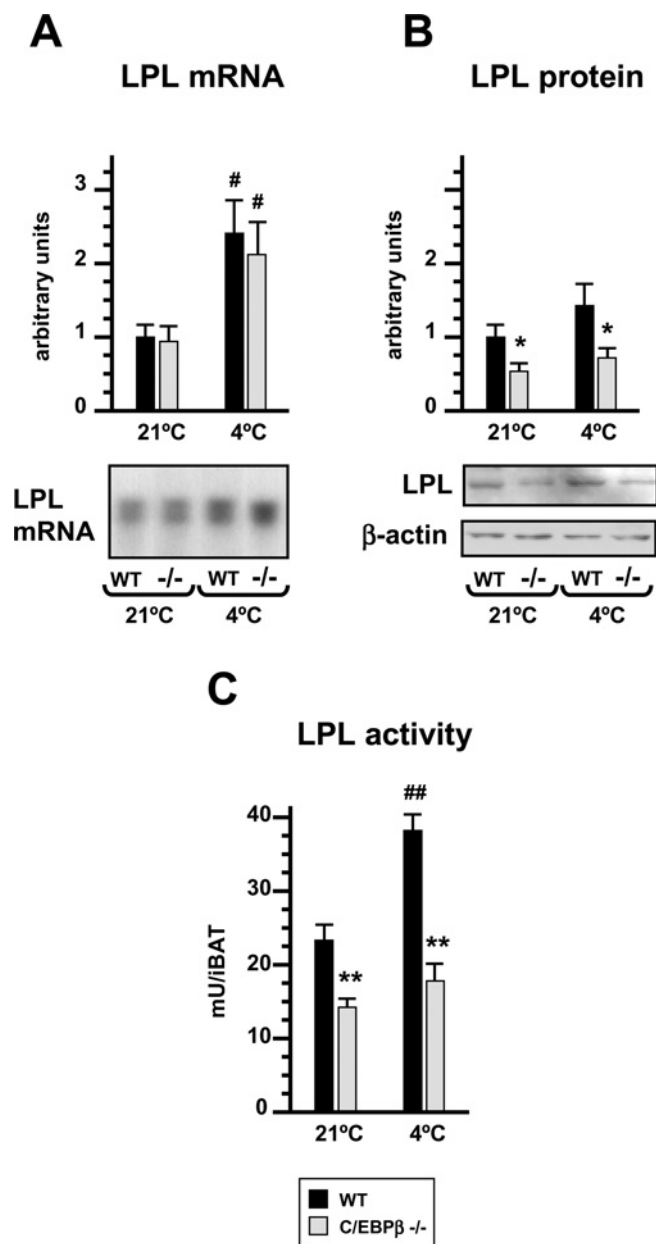
The expression of genes described to be targets of C/EBP-dependent regulation of transcription *in vitro*, such as PEPCK and GLUT4 [39,40], is unaffected in BAT and primary brown adipocytes of C/EBP $\beta^{-/-}$  mice. Furthermore, the expression of UCP1 is

highly increased in response to cold and in primary brown adipocytes lacking C/EBP $\beta$ . Neither C/EBP $\alpha$  nor C/EBP $\delta$  are over-expressed in compensation for the lack of C/EBP $\beta$ , indicating either that C/EBP $\beta$  is not required to regulate these genes or that C/EBP $\alpha$  and/or C/EBP $\delta$  may be redundant to C/EBP $\beta$  at their normal levels of expression. However, expression of C/EBP $\delta$  is significantly induced by cold in C/EBP $\beta^{-/-}$  BAT and also by noradrenaline treatment in primary brown adipocytes, suggesting that C/EBP $\delta$  mediates the activation of UCP1 gene expression. Thus different sensitivity to C/EBP $\delta$  and/or LIP (see below) may explain the different behaviour observed in the expression of the C/EBP-target genes in BAT.

Similar behaviour to UCP1 has been described for another C/EBP $\beta$ -target gene, the interleukin-6 gene, whose gene expression is enhanced by deletion of the gene for C/EBP $\beta$  [41]. Since C/EBP $\beta$ -null mice lack both C/EBP $\beta$  isoforms, LAP and LIP, the absence of dominant-negative LIP may have a stronger effect than that of active LAP. This is especially relevant in brown fat, which is characterized by high levels of LIP. Furthermore, the ratio of LAP/LIP in BAT has been reported to increase in physiological situations of UCP1 gene expression induction, such as acute cold exposure and perinatal development [22,23]. The dominant-negative role of LIP upon regulation of the transcription of the UCP1 gene promoter reported in the present paper supports this hypothesis further.

In view of the above results, other mechanisms that control thermogenesis may be affected in the BAT of C/EBP $\beta$ -null mice, in agreement with other experimental models of defective thermoregulation. Thus genetically obese *ob/ob* and *db/db* mice are also sensitive to the cold, although the expression of the UCP1 gene is normally induced during exposure to cold [42]. Furthermore, mice with inherited defects of fatty acid oxidation show abnormal non-shivering thermogenesis and BAT function, resulting in sensitivity to cold [43]. The same cold-intolerant phenotype is found in mice with a targeted inactivation of the gene for the  $\beta$ -oxidation enzyme long-chain acyl-CoA dehydrogenase [43], indicating that defects in BAT lipid metabolism can render mice cold-sensitive.

Our findings indicate that, although the mobilization of stored substrates is normally induced, the overall supply of endogenous fatty acids for thermogenesis is reduced in BAT of C/EBP $\beta^{-/-}$  mice. This reduction is due to the lower triacylglycerol content. The capacity of C/EBP $\beta^{-/-}$  mice to accumulate lipid in BAT during foetal life is also impaired [24]. Since C/EBP $\alpha$  and PPAR $\gamma$  are normally expressed in brown fat of foetal [24] and adult (present study) C/EBP $\beta^{-/-}$  mice, a specific role for C/EBP $\beta$  in controlling lipid metabolism in developing BAT can be suggested.



**Figure 6** Effects of cold exposure on LPL gene expression and activity in brown fat from wild-type and C/EBPβ<sup>-/-</sup> mice

(A) LPL mRNA analysis. A representative Northern blot analysis is depicted beneath the histogram. For experimental and Figure details see the legend to Figure 1. (B) Immunoblot analysis of cytosolic/membrane protein extracts isolated from interscapular BAT from wild-type (WT) or C/EBPβ<sup>-/-</sup> mice, exposed or not to 4°C for 4 h. Detected LPL and actin proteins in a representative Western blot analysis are shown beneath the histogram. Results are representative of three separate experiments. (C) LPL activity in interscapular BAT from control or cold-exposed wild-type (WT) or C/EBPβ<sup>-/-</sup> mice as described above. Results are means ± S.E.M. of six to eight animals per group. Statistical significance of comparison between genotypes is shown as \*\**P* ≤ 0.01, and between control and cold-exposed mice as #*P* ≤ 0.05, ##*P* ≤ 0.01.

In addition to the endogenous supply of fuel for BAT thermogenesis, an increase in the provision of circulating substrates to this tissue is also required during cold exposure. This external supply involves (i) an increase in the uptake and utilization of circulating triacylglycerols due to the induction of LPL activity in BAT (see below), and (ii) mobilization of stored substrates, mainly glycogen in liver to release glucose, and triacylglycerols in WAT

to release non-esterified fatty acids. In contrast with previously reported defective glycogen mobilization in liver and impaired lipolysis in WAT of C/EBPβ<sup>-/-</sup> mice in response to starvation [13] and during diabetes [14], changes in circulating metabolites indicate that substrate mobilization in C/EBPβ<sup>-/-</sup> liver and WAT is unaltered in response to cold.

In response to cold exposure, LPL activity is rapidly stimulated in BAT through adrenergic pathways [44]. This involves an increase in LPL mRNA expression [45,46], but also translational and/or post-translational regulation [45] in the stimulation of LPL activity. In contrast, LPL in WAT is under negative adrenergic control [37], and a mechanism of regulation of translation by adrenaline through *trans*-acting proteins interacting with the 3'-untranslated region of the LPL mRNA has been characterized [47]. C/EBPβ<sup>-/-</sup> mice show reduced basal LPL activity in BAT, but not in WAT, heart or skeletal muscle. Cold exposure causes a similar increase in LPL mRNA in BAT of C/EBPβ<sup>-/-</sup> and wild-type mice, indicating a normal response to adrenergic stimulation in C/EBPβ<sup>-/-</sup> BAT. However, the concomitant rise in LPL activity in the BAT owing to cold is impaired in C/EBPβ<sup>-/-</sup> mice, which is not due to an increased release of LPL into blood. In contrast, the amount of LPL protein correlates with LPL activity, indicating that the lack of C/EBPβ specifically affects the post-transcriptional regulation of LPL activity in BAT. A tissue-specific control of LPL mRNA translation in BAT has been observed recently [48]. Moreover, a distinct type of biological effects of C/EBPβ mediated by protein-protein interactions and unrelated to transcription has been described previously (reviewed in [49]). The direct or indirect mechanism by which C/EBPβ is involved in BAT-specific regulation of LPL gene translation deserves further investigation.

In summary, reduced basal LPL activity can contribute to lower triacylglycerol storage in C/EBPβ<sup>-/-</sup> BAT, whereas the impairment in LPL activity during cold exposure results in defective supply of fatty acids as fuel for thermogenesis in BAT. This defective exogenous supply, together with the reduced endogenous supply of fatty acids, can impair thermogenesis in BAT. In addition to their role as major substrates for oxidation, fatty acids also have another relevant function in non-shivering thermogenesis: they activate the uncoupling activity of UCP1 by directly interacting with the protein in the inner membrane of mitochondria [18]. Thus, together with the failure to provide fuel for thermogenic activity, the impaired activation of UCP1 by fatty acids can also contribute to the defective thermoregulation in C/EBPβ<sup>-/-</sup> mice associated with impaired lipid metabolism.

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## REFERENCES

- 1 Lekstrom-Himes, J. and Xanthopoulos, K. G. (1998) Biological role of the CCAAT/enhancer-binding protein family of transcription factors. *J. Biol. Chem.* **273**, 28545–28548.
- 2 Cao, Z., Umek, R. M. and McKnight, S. L. (1991) Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes Dev.* **5**, 1538–1552.
- 3 Roesler, W. J. (2001) The role of C/EBP in nutrient and hormonal regulation of gene expression. *Annu. Rev. Nutr.* **21**, 141–165.

- 4 Poli, V., Mancini, F. P. and Cortese, R. (1990) IL-6DBP, a nuclear protein involved in interleukin-6 signal transduction, defines a new family of leucine zipper proteins related to C/EBP. *Cell* **63**, 643–653
- 5 Descombes, P., Chojkier, M., Lichtsteiner, S., Falvey, E. and Schibler, U. (1990) LAP, a novel member of the C/EBP gene family, encodes a liver-enriched transcriptional activator protein. *Genes Dev.* **4**, 1541–1551
- 6 Akira, S., Isshiki, H., Sugita, T., Tanabe, O., Kinoshita, S., Nishio, Y., Nakajima, T., Hirano, T. and Kishimoto, T. (1990) A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. *EMBO J.* **9**, 1897–1906
- 7 Calkhoven, C. F., Müller, C. and Leutz, A. (2000) Translational control of C/EBP $\alpha$  and C/EBP $\beta$  isoform expression. *Genes Dev.* **14**, 1920–1932
- 8 Descombes, P. and Schibler, U. (1991) A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. *Cell* **67**, 569–579
- 9 Screpanti, I., Romani, L., Musiani, P., Modesti, A., Fattori, E., Lazzaro, D., Sellitto, C., Scarpa, S., Bellavia, D., Lattanzio, G. et al. (1995) Lymphoproliferative disorder and imbalanced T-helper response in C/EBP $\beta$ -deficient mice. *EMBO J.* **14**, 1932–1941
- 10 Tanaka, T., Akira, S., Yoshida, K., Umemoto, M., Yoneda, Y., Shirafuji, N., Fujiwara, H., Suematsu, S., Yoshida, N. and Kishimoto, T. (1995) Targeted disruption of the NF-IL6 gene discloses its essential role in bacteria killing and tumor cytotoxicity by macrophages. *Cell* **80**, 353–361
- 11 Croniger, C., Trus, M., Lysek-Stupp, K., Cohen, H., Liu, Y., Darlington, G. J., Poli, V., Hanson, R. W. and Reshef, L. (1997) Role of the isoforms of CCAAT/enhancer-binding protein in the initiation of phosphoenolpyruvate carboxykinase (GTP) transcription at birth. *J. Biol. Chem.* **272**, 26306–26312
- 12 Croniger, C. M., Millward, C., Yang, J., Kawai, Y., Arinze, I. J., Liu, S., Harada-Shiba, M., Chakravarty, K., Friedman, J. E., Poli, V. and Hanson, R. W. (2001) Mice with a deletion in the gene for CCAAT/enhancer-binding protein  $\beta$  have an attenuated response to cAMP and impaired carbohydrate metabolism. *J. Biol. Chem.* **276**, 629–638
- 13 Liu, S., Croniger, C., Arizmendi, C., Harada-Shiba, M., Ren, J., Poli, V., Hanson, R. W. and Friedman, J. E. (1999) Hypoglycemia and impaired hepatic glucose production in mice with a deletion of the C/EBP  $\beta$  gene. *J. Clin. Invest.* **103**, 207–213
- 14 Arizmendi, C., Liu, S., Croniger, C., Poli, V. and Friedman, J. E. (1999) The transcription factor CCAAT/enhancer-binding protein  $\beta$  regulates gluconeogenesis and phosphoenolpyruvate carboxykinase (GTP) gene transcription during diabetes. *J. Biol. Chem.* **274**, 13033–13040
- 15 Wang, L., Shao, J., Muhlenkamp, P., Liu, S., Klepcyk, P., Ren, J. and Friedman, J. E. (2000) Increased insulin receptor substrate-1 and enhanced skeletal muscle insulin sensitivity in mice lacking CCAAT/enhancer-binding protein  $\beta$ . *J. Biol. Chem.* **275**, 14173–14181
- 16 Lowell, B. B., Susulic, V., Hamann, A., Lawitts, J. A., Himms-Hagen, J., Boyer, B. B., Kozak, L. P. and Flier, J. S. (1993) Development of obesity in transgenic mice after genetic ablation of tissue. *Nature (London)* **366**, 740–742
- 17 Ricquier, D. and Bouillaud, F. (2000) Mitochondrial uncoupling proteins: from mitochondria to the regulation of energy balance. *Biochem. J.* **345**, 161–179
- 18 Nicholls, D. G., Cunningham, S. A. and Rial, E. (1986) The bioenergetic mechanisms of brown adipose tissue mitochondria. In *Brown Adipose Tissue* (Trayhurn, P. and Nicholls, D. G., eds.), pp. 52–85, Edward Arnold Publishers, London
- 19 Enerbäck, S., Jacobsson, A., Simpson, E. M., Guerra, C., Yamashita, H., Harper, M. E. and Kozak, L. P. (1997) Mice lacking mitochondrial uncoupling protein are cold-sensitive but not obese. *Nature (London)* **387**, 90–94
- 20 Yubero, P., Barberà, M. J., Alvarez, R., Viñas, O., Mampel, T., Iglesias, R., Villarroya, F. and Giral, M. (1998) Dominant negative regulation by c-Jun of transcription of the uncoupling protein-1 gene through a proximal cAMP-regulatory element: a mechanism for repressing basal and noradrenaline-induced expression of the gene before brown adipocyte differentiation. *Mol. Endocrinol.* **12**, 1023–1037
- 21 Yubero, P., Manchado, C., Cassard-Doulcier, A. M., Mampel, T., Viñas, O., Iglesias, R., Giral, M. and Villarroya, F. (1994) CCAAT/enhancer binding proteins  $\alpha$  and  $\beta$  are transcriptional activators of the brown fat uncoupling protein gene promoter. *Biochem. Biophys. Res. Commun.* **198**, 653–659
- 22 Manchado, C., Yubero, P., Viñas, O., Iglesias, R., Villarroya, F., Mampel, T. and Giral, M. (1994) CCAAT/enhancer-binding proteins  $\alpha$  and  $\beta$  in brown adipose tissue: evidence for a tissue-specific pattern of expression during development. *Biochem. J.* **302**, 695–700
- 23 Carmona, M. C., Iglesias, R., Obregón, M. J., Darlington, G. J., Villarroya, F. and Giral, M. (2002) Mitochondrial biogenesis and thyroid status maturation in brown fat require CCAAT/enhancer-binding protein  $\alpha$ . *J. Biol. Chem.* **277**, 21489–21498
- 24 Tanaka, T., Yoshida, N., Kishimoto, T. and Akira, S. (1997) Defective adipocyte differentiation in mice lacking the C/EBP $\beta$  and/or C/EBP $\delta$  gene. *EMBO J.* **16**, 7432–7443
- 25 Alvarez, R., de Andrés, J., Yubero, P., Viñas, O., Mampel, T., Iglesias, R., Giral, M. and Villarroya, F. (1995) A novel regulatory pathway of brown fat thermogenesis: retinoic acid is a transcriptional activator of the mitochondrial uncoupling protein gene. *J. Biol. Chem.* **270**, 5666–5673
- 26 Barberà, M. J., Schlüter, A., Pedraza, N., Iglesias, R., Villarroya, F. and Giral, M. (2001) Peroxisome proliferator-activated receptor  $\alpha$  activates transcription of the brown fat uncoupling protein-1 gene: a link between regulation of the thermogenic and lipid oxidation pathways in the brown fat cell. *J. Biol. Chem.* **276**, 1486–1493
- 27 Hara, A. and Radin, N. S. (1978) Lipid extraction of tissues with a low-toxicity solvent. *Anal. Biochem.* **90**, 420–426
- 28 Prunet-Marcassus, B., Moulin, K., Carmona, M. C., Villarroya, F., Penicaud, L. and Castella, L. (1999) Inverse distribution of uncoupling proteins expression and oxidative capacity in mature adipocytes and stromal-vascular fractions of rat white and brown adipose tissues. *FEBS Lett.* **464**, 184–188
- 29 Ramírez, I., Kryski, A. J., Ben-Zeev, O., Schotz, M. C. and Severson, D. L. (1985) Characterization of triacylglycerol hydrolase activities in isolated myocardial cells from rat heart. *Biochem. J.* **232**, 229–236
- 30 Ricquier, D., Bouillaud, F., Toumelin, P., Mory, G., Bazin, R., Arch, J. and Penicaud, L. (1986) Expression of uncoupling protein mRNA in thermogenic or weakly thermogenic brown adipose tissue: evidence for a rapid  $\beta$ -adrenoreceptor-mediated and transcriptionally regulated step during activation of thermogenesis. *J. Biol. Chem.* **261**, 13905–13910
- 31 Jacobsson, A., Muhleisen, M., Cannon, B. and Nedergaard, J. (1994) The uncoupling protein thermogenin during acclimation: indications for pretranslational control. *Am. J. Physiol.* **267**, R999–R1007
- 32 Carmona, M. C., Valmaseda, A., Brun, S., Viñas, O., Mampel, T., Iglesias, R., Giral, M. and Villarroya, F. (1998) Differential regulation of uncoupling protein-2 and uncoupling protein-3 gene expression in brown adipose tissue during development and cold exposure. *Biochem. Biophys. Res. Commun.* **243**, 224–228
- 33 Arsenijevic, D., Onuma, H., Pecqueur, C., Raimbault, S., Manning, B. S., Miroux, B., Couplan, E., Alves-Guerra, M. C., Gubern, M., Surwit, R. et al. (2000) Disruption of the uncoupling protein-2 gene in mice reveals a role in immunity and reactive oxygen species production. *Nat. Genet.* **26**, 435–439
- 34 Pecqueur, C., Alves-Guerra, M. C., Gelly, C., Levi-Meyrueis, C., Couplan, E., Collins, S., Ricquier, D., Bouillaud, F. and Miroux, B. (2001) Uncoupling protein 2, *in vivo* distribution, induction upon oxidative stress, and evidence for translational regulation. *J. Biol. Chem.* **276**, 8705–8712
- 35 Puigserver, P., Wu, Z., Park, C. W., Graves, R., Wright, M. and Spiegelman, B. M. (1998) A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell* **92**, 829–839
- 36 Lindgren, E. M., Nielsen, R., Petrovic, N., Jacobsson, A., Mandrup, S., Cannon, B. and Nedergaard, J. (2004) Noradrenaline represses PPAR (peroxisome-proliferator-activated receptor)  $\gamma$ 2 gene expression in brown adipocytes: intracellular signalling and effects on PPAR $\gamma$ 2 and PPAR $\gamma$ 1 protein levels. *Biochem. J.* **382**, 597–606
- 37 Enerbäck, S. and Gimble, J. M. (1983) Lipoprotein lipase gene expression: physiological regulators at the transcriptional and post-transcriptional level. *Biochim. Biophys. Acta* **1169**, 107–125
- 38 Thomas, S. A. and Palmiter, R. D. (1997) Thermoregulatory and metabolic phenotypes of mice lacking noradrenaline and adrenaline. *Nature (London)* **387**, 94–97
- 39 Park, E. A., Gurney, A. L., Nizelski, S. E., Hakimi, P., Cao, Z., Moorman, A. and Hanson, R. W. (1993) Relative roles of CCAAT/enhancer-binding protein  $\beta$  and cAMP regulatory element-binding protein in controlling transcription of the gene for phosphoenolpyruvate carboxykinase (GTP). *J. Biol. Chem.* **268**, 613–619
- 40 Kaestner, K. H., Christy, R. J. and Lane, M. D. (1990) Mouse insulin-responsive glucose transporter gene: characterization of the gene and *trans*-activation by the CCAAT/enhancer binding protein. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 251–255
- 41 Fattori, E., Sellitto, C., Cappelletti, M., Lazzaro, D., Bellavia, D., Screpanti, I., Gulino, A., Costantini, F. and Poli, V. (1995) Functional analysis of IL-6 and IL-6DBP/C/EBP  $\beta$  by gene targeting. *Ann. N.Y. Acad. Sci.* **762**, 262–273
- 42 Trayhurn, P. and James, W. P. (1978) Thermoregulation and non-shivering thermogenesis in the genetically obese (*ob/ob*) mouse. *Pflügers Arch.* **373**, 189–193
- 43 Guerra, C., Kozak, R. A., Walsh, K., Kurtz, D. M., Wood, P. A. and Kozak, L. P. (1998) Abnormal nonshivering thermogenesis in mice with inherited defects of fatty acid oxidation. *J. Clin. Invest.* **102**, 1724–1731
- 44 Carneheim, C., Nedergaard, J. and Cannon, B. (1984)  $\beta$ -Adrenergic stimulation of lipoprotein lipase in rat brown adipose tissue during acclimation to cold. *Am. J. Physiol.* **246**, E327–E333
- 45 Giral, M., Martín, I., Vilaró, S., Villarroya, F., Mampel, T., Iglesias, R. and Viñas, O. (1990) Lipoprotein lipase mRNA expression in brown adipose tissue: translational and/or posttranslational events are involved in the modulation of enzyme activity. *Biochim. Biophys. Acta* **1048**, 270–273

- 46 Mitchell, J. R., Jacobsson, A., Kirchgessner, T. G., Schotz, M. C., Cannon, B. and Nedergaard, J. (1992) Regulation of expression of the lipoprotein lipase gene in brown adipose tissue. *Am. J. Physiol.* **263**, E500–E506
- 47 Ranganathan, G., Phan, D., Pokrovskaya, I. D., McEwen, J. E., Li, C. and Kern, P. A. (2002) The translational regulation of lipoprotein lipase by epinephrine involves an RNA binding complex including the catalytic subunit of protein kinase A. *J. Biol. Chem.* **277**, 43281–43287
- 48 Hensley, L. L., Ranganathan, G., Wagner, E. M., Wells, B. D., Daniel, J. C., Vu, D., Semenkovich, C. F., Zechner, R. and Kern, P. A. (2003) Transgenic mice expressing lipoprotein lipase in adipose tissue: absence of the proximal 3'-untranslated region causes translational up-regulation. *J. Biol. Chem.* **278**, 32702–32709
- 49 McKnight, S. L. (2001) McBindall – a better name for CCAAT/enhancer binding proteins? *Cell* **107**, 259–261

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